

Delineation of the Amino Acid Residues Involved in Transcytosis and Catabolism of Mouse IgG1¹

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The MHC class I-related receptor, FcRn, is involved in both the transcytosis of serum γ -globulins (IgGs) and in regulating their serum persistence. The interaction site of FcRn on the Fc region of rodent IgG has been mapped to residues at the CH2-CH3 domain interface using site-directed mutagenesis and x-ray crystallographic analyses. In the current study, the role of individual residues (H310, H433, and N434) at this interface in mediating the Fc-FcRn interaction has been investigated using recombinant, mutated Fc hinge fragments derived from mouse IgG1. In addition, two highly conserved Fc histidines (H435 and H436) have been mutated to alanine, and the resulting mutated Fc hinge fragments were analyzed in both transcytosis and pharmacokinetic studies in mice and in competition binding assays using recombinant, soluble FcRn. The analyses indicate that mutation of H310, H435, and, to a lesser extent, H436 to alanine results in reduced activity of the Fc hinge fragments in both in vivo and in vitro assays. Thus, in addition to the previously defined role of I253 in the FcRn-IgG interaction, these histidines play a key role in mediating the functions conducted by this Fc receptor. The effects of these mutations on binding of Fc hinge fragments to staphylococcal protein A have also been analyzed and demonstrate a partial, but not complete, overlap of the FcRn and staphylococcal protein A interaction sites on mouse IgG1. *The Journal of Immunology*, 1997, 158: 2211–2217.

Recent studies have indicated that for mouse IgG, the MHC class I-related receptor FcRn⁴ is involved in both the control of serum half-life and transcytosis across neonatal intestinal/maternofetal barriers (1–3). Site-directed mutagenesis has been used to identify amino acid residues of mouse IgG1 (mIgG1) that regulate these processes (4–7). These residues are located at the CH2-CH3 domain interface and are highly conserved in both human and murine IgGs (8). To identify these amino acids, recombinant Fc hinge fragments with double mutations of amino acid residues in the CH2 domain (H310A/Q311N) and CH3 domain (H433A/N434Q), and a single mutation in the CH2 domain (I253A) have been characterized in pharmacokinetic studies (4, 5) and in transcytosis across yolk sac and neonatal small intestine (6, 7). These studies demonstrated that mutation of the CH2 domain residues had a more marked effect on both serum half-life and transcytosis than the CH3 domain mutations. Furthermore, the mutants were also impaired in binding to recombinant,

soluble FcRn (9). However, these analyses did not exclude a role for other conserved amino acid residues in the CH3 domain. In addition, for the H310A/Q311N and H433A/N434Q mutants, two simultaneous mutations were made in both the CH2 and CH3 domains, and it was, therefore, not possible to identify which of the four amino acids (H310, Q311, H433, and N434) are directly involved in building the FcRn interaction site of mIgG1. The aim of this work was to identify which of these four amino acid residues are involved in the regulation of IgG transcytosis/catabolism and to investigate a possible role for other CH3 domain residues (H435 and H436) that are highly conserved in IgGs (8).

Materials and Methods

Generation of mutated Fc hinge fragments derived from mIgG1

Mutations were made using designed mutagenic oligonucleotides and either splicing by overlap extension (10) or site-directed mutagenesis (11, 12). The mutants are described in Table I, and the generation of mutants I253A and H285A has been described previously (4, 5). For other mutants, mutagenic oligonucleotides used in site-directed mutagenesis were as follows: H433A, 5'-GGTGGTTGGCCAGGCCCT-3'; H435A, 5'-CAGTATGGCCGTTGTGCA-3'; and H436A, 5'-CTCAGTAGCGTGGTTGTG-3'. Mutants H310A, N434A, and N434Q were made using splicing by overlap extension (10) with the following mutagenic oligonucleotides: H310A, 5'-CCCATCATGGCCAGGACTGG-3' and 5'-CCAGTCCTGGGCATGATGGG-3'; N434A, 5'-GGCTGCACGCCGACCATACT-3' and 5'-AGTATGGTGGCGGTGCAGGCCCTC-3'; and N434Q, 5'-AGTATGTGTTGGTGCAG-3' and 5'-CTGCACCAACACCATACT-3'. For each oligonucleotide, percent underlining indicates mutated bases. For all mutants, the corresponding genes were sequenced using the dideoxynucleotide method (13) and Sequenase before functional analysis.

Expression and purification of the recombinant proteins

Wild-type (wt) and mutant Fc hinge fragments tagged with carboxyl-terminal hexahistidine peptides were purified using Ni²⁺-NTA-agarose (Qia-gen, Chatsworth, CA) as described previously (4). After dialysis against 15 mM phosphate buffer/50 mM NaCl, pH 7.5, the mutants were either kept at 4°C for short term storage (<10 days) or freeze dried for longer term storage. Recombinant soluble mouse FcRn was expressed and purified using the baculovirus system as described previously (9) and stored at 4°C.

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⁴ Abbreviations used in this paper: FcRn, neonatal Fc receptor; Fc, immunoglobulin constant region fragment; mIgG1, mouse immunoglobulin G1; wt, wild type; CD, circular dichroism; PB-6, 50 mM phosphate buffer with 250 mM NaCl and 5 mM Na₂EDTA, pH 6.0; SpA, staphylococcal protein A; PB-7.5, 50 mM phosphate buffer containing 250 mM NaCl, 5 mM Na₂EDTA, pH 7.5.

Table I. Recombinant Fc-hinge derivatives used in this study

Designation	Mutation	Domain
WT Fc-hinge	None	
I253A ^a	Ile 253 to Ala	CH2
H285A	His 285 to Ala	CH2
H310A	His 310 to Ala	CH2
H433A	His 433 to Ala	CH3
H433A/N434Q ^a	His 433 to Ala and Asn 434 to Gln	CH3
N434A	Asn 434 to Ala	CH3
N434Q	Asn 434 to Gln	CH3
H435A	His 435 to Ala	CH3
H436A	His 436 to Ala	CH3

^a Mutants described previously (4, 5).

Analysis of the mutant Fc hinge fragments using SDS-PAGE and circular dichroism (CD)

SDS-PAGE (14) and CD analyses were conducted as described previously (4).

Radiolabeling of the proteins

Monoclonal mIgG1, recombinant mouse Fc γ hinge fragments, and recombinant mouse FcRn (mFcRn) (9) were radiolabeled with [¹²⁵I]Na (Amersham, Arlington Heights, IL) using the Iodogen reagent (15) as described previously (4). Free iodine was removed by centrifugation on MicroSpin G-25 columns (Pharmacia, Piscataway, NJ). The specific activities of the radiolabeled proteins were approximately 5×10^6 cpm/ μ g, with <5% free iodine. The radioactive proteins were stored at 4°C for not more than 1 wk before injection into mice.

Chromatographic analysis

All radiolabeled Fc hinge fragments were analyzed on an s-250 column (Bio-Rad, Hercules, CA) by permeation HPLC. The sera collected from mice injected with radiolabeled Fc hinge fragments at 24 h were pooled and analyzed by HPLC on an s-250 column (Bio-Rad). The radioactivities of the chromatographic fractions were measured with a gamma counter, and the molecular mass and heterogeneity of the radioactive peak were determined.

Determination of serum IgG concentration

The concentration of serum IgG was determined using radial immunodiffusion with Nanorid and Bindarid kits (The Binding Site, Birmingham, UK). Precipitin ring diameters were measured electronically.

Pharmacokinetic analyses

Pharmacokinetics of radioiodinated Fc hinge fragments were determined in 6-wk-old BALB/c mice (Harlan Sprague-Dawley Laboratory, Indianapolis, IN) as described previously (4, 5).

Maternofetal transmission

Previously described methodology (7) was used with pregnant outbred SCID mice (Taconic Co., Germantown, NY) near term (15–18 days). In brief, mice were fed 0.01% NaI in drinking water and then 1 day later injected with radiolabeled protein (2×10^7 to 5×10^7 cpm) in the tail vein. Mice were bled with a 20- μ l capillary 3 min postinjection, and 24 h later fetuses were delivered by cesarean section. The fetuses of a litter were pooled (discarding the placenta), washed in saline, weighed, frozen in liquid nitrogen, and homogenized in 10 vol of 10% TCA. The suspension was centrifuged, and the radioactivity of the precipitate was determined in a gamma counter. The percentage of transmission was calculated with the formula: % transmission (%T) = $(R_3)/(R_1 - R_2) \times (W \times 0.72)/0.02$, where R1 is radioactivity in maternal blood at 3 min, R2 is radioactivity in maternal blood at 24 h, W is body weight (grams), and R3 is radioactivity of the fetuses.

The total weight and number of fetuses in a given litter varied from litter to litter, and therefore, the transmission data are presented per unit weight of fetuses rather than the amount transferred per litter (%T/g) (7). The blood volume of pregnant mice was considered to be 7.2% of body weight (16). The radioactivity in the maternal blood available for transmission to

the fetus was calculated by deducting the radioactivity remaining at 24 h from that measured at 3 min after the injection of radiolabeled protein.

Inhibition of transintestinal transfer

BALB/c neonatal mice (10–14 days old) from the Animal Resource Center, University of Texas Southwestern Medical Center (Dallas, TX), were intubated with a mixture of [¹²⁵I]mIgG1 and Fc hinge fragment at a Fc/IgG molar ratio of approximately 2000 as described previously (6). The percentage of inhibition was calculated relative to the transfer of the same amount of [¹²⁵I]mIgG1 without inhibitor.

Inhibition of FcRn binding to mIgG1-Sepharose

All Fc hinge derivatives were dialyzed into 50 mM phosphate buffer with 250 mM NaCl and 5 mM Na₂EDTA, pH 6.0 (PB-6), and adjusted to a concentration of 1 mg/ml. Three hundred microliters of Fc hinge (wt or mutant) or PB-6 was incubated in Eppendorf tubes with rotation for 30 min at 25°C with 150 μ l of mIgG1-Sepharose (1 mg/ml packed gel, 50% suspension), 50 μ l of PB-6 containing 10 mg/ml OVA (Sigma Chemical Co., St. Louis, MO), and 10 μ l of [¹²⁵I]FcRn (0.1 μ g/200,000 cpm). Following incubation, 500 μ l of ice-cold PB-6 was added, and the gel was washed three times by centrifugation at $12,000 \times g$ for 3 min using ice-cold PB-6 (plus 1 mg/ml OVA). The radioactivity bound to the gel was determined. The gel pellet was resuspended in 1 ml of PB-7.5 (with 1 mg/ml OVA), and the supernatant was discarded after centrifugation. The remaining radioactivity bound to the gel was determined. The radioactivity specifically bound to the mIgG1-Sepharose gel was calculated by subtracting the remaining radioactivity from the bound radioactivity. The inhibition of binding of FcRn to mIgG1-Sepharose by Fc derivatives was calculated using the equation: % inhibition = $100 - 100 A/B$, where A is the specific radioactivity bound to mIgG1-Sepharose in the presence of Fc hinge fragment, and B is the specific radioactivity bound in the absence of Fc hinge fragment.

Analysis of binding to staphylococcal protein A (SpA)

SpA-agarose gel (0.5 ml) was equilibrated with PB-7.5 and 1 mg/ml OVA (PB-7.5). Fifty to one hundred microliters of each [¹²⁵I]-labeled Fc hinge fragment containing 50 μ g of protein was loaded onto the column, incubated for 15 min, and then washed with 10 column volumes of the same buffer. Bound Fc hinge fragments were eluted with 100 mM acetic acid. The amounts of radioactivity in the flow-through, washes, and eluates were determined. The ratio of bound/unbound was calculated, and the percentage of binding of each mutant relative to the wt Fc hinge fragment was determined.

Results

Expression and analysis of mouse Fc γ hinge mutants

Plasmids encoding the wt Fc hinge and mutants (Table I) were constructed, and the proteins were expressed and purified using *Escherichia coli* as a host. With the exception of the H285A mutant, the residues that have been mutated are all in close proximity to the CH2-CH3 domain interface (17) and are also highly conserved in the IgG isotypes of both mouse and man (8). As described previously, the radiolabeled Fc hinge derivatives emerged essentially as single peaks with a retention time corresponding to 55 kDa when analyzed on an s-250 column (4) (data not shown). Taken together with HPLC analyses, reducing and nonreducing SDS-PAGE analyses indicate that the Fc hinge derivatives are expressed as a mixture of noncovalently linked and sulfhydryl-linked homodimers (Fig. 1). In addition, CD studies of the Fc hinge derivatives show that the mutations do not result in large scale changes in the structures of the recombinant proteins (Fig. 2).

Pharmacokinetic analysis of the Fc hinge fragments

Radiolabeled Fc hinge fragments were injected into mice, and the serum radioactivity was monitored at various time points following injection. For each Fc hinge derivative, the elimination curves in different mice were similar, and Figure 3 shows representative curves for one mouse from each group.

For each recombinant Fc hinge fragment, the serum samples collected at the 24 h point from mice within one group were pooled

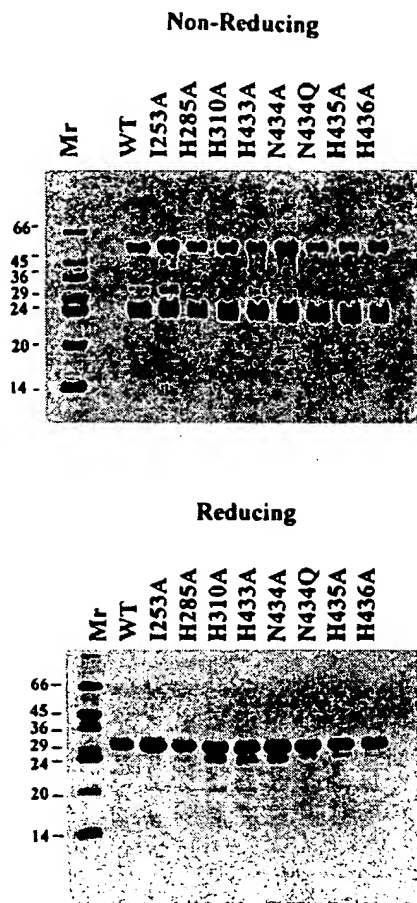


FIGURE 1. SDS-PAGE of wt and mutant Fc hinge fragments using nonreducing and reducing conditions. The molecular mass of size standards (Mr) are indicated on the left margins.

and subjected to HPLC on an μ -250 column. For all the Fc hinge fragments, the majority of the radioactivity eluted as a single peak with a retention time corresponding to the molecular mass of the injected protein (55 kDa; data not shown). In agreement with our previous results (4), this indicates that the Fc hinge derivatives persist in serum as homodimeric molecules and are not proteolytically digested or associated with other serum proteins. The pharmacokinetic parameters of the Fc hinge derivatives are shown in Table II, and the α -phase represents the equilibration time between the intra- and extravascular space, whereas the β -phase represents the elimination of the equilibrated protein from the intravascular space. Furthermore, during the α -phase, any misfolded protein molecules that might be present in the recombinant protein preparations are eliminated (C. Medesan, unpublished observations), and therefore, the β -phase represents the elimination of correctly folded protein from the intravascular space.

The data clearly demonstrate that some mutations have a significant effect on the β -phase half-life of the corresponding Fc hinge fragment. Thus, mutation of H310 in the CH2 domain has a marked effect on the catabolic rate (Table II). In contrast, mutation of H285, located in a loop on the external surface of the CH2 domain distal to the CH2-CH3 interface (17), has no effect on the catabolic rate, and this is consistent with our previous findings (5). Simultaneous mutation of H433 and N434 decreases the β -phase half-life to 77 h (36% decrease), while single mutation at each of these positions yielded two mutants (H433A and N434A) with the same half-life as the wt Fc hinge. Substitution of N434 with glu-

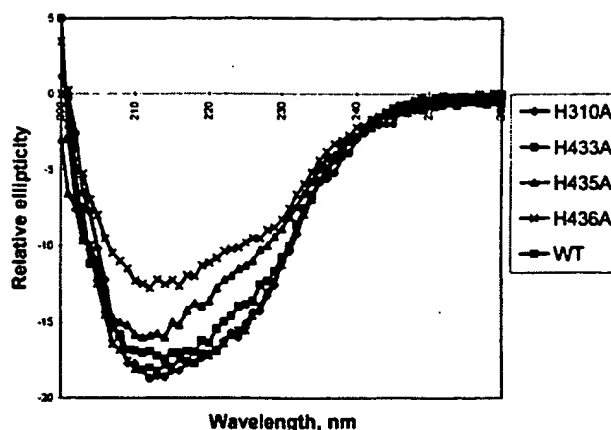


FIGURE 2. CD spectra of recombinant Fc hinge fragments.

tamine instead of alanine (N434Q) also yielded an Fc hinge fragment with a half-life similar to that of the wt Fc hinge (Table II). The half-life of the H433A-N434Q mutant (76.9 h) is greater than the value reported previously (50.3 h) (4), and this is also observed for the wt Fc hinge fragment (119 vs 82.9 h), H285A (106 vs 85 h), and I253A (26 vs 20 h). An explanation for these apparent discrepancies is that the BALB/c mice used in the present work (from Harlan Laboratories) have an IgG concentration of 1.0 ± 0.4 mg/ml (average of 25 mice), which is considerably lower than that in BALB/c mice from our own animal colony (4.6 ± 0.8 mg/ml) used in earlier experiments (4, 5). The concentration-catabolism relation (18) predicts that the half-life of IgG will be longer in mice with lower serum IgG concentrations, and this may explain the longer half-lives of the Fc hinge fragments in the cases above where direct comparisons have been made.

Mutation of H435 in the CH3 domain has an effect as marked as that induced by mutation of I253 or H310 in the CH2 domain, clearly indicating that this CH3 domain residue plays an important role in building the catabolic site of mIgG1. Furthermore, the H436A mutant has a half-life of 49 h, demonstrating that H436 plays a more minor role than I253, H310, or H435 in controlling catabolism (Table II).

Maternofetal transfer

The analysis of the pharmacokinetics of the Fc hinge fragments was extended to maternofetal transfer studies. The transfer of radiolabeled Fc hinge derivatives from the circulation of near-term pregnant SCID mice to the fetuses was analyzed by measuring the protein-bound radioactivity taken up by fetuses of one litter relative to the radioactivity present in the maternal blood during the 24-h interval used for the transfer experiment. The results are shown in Figure 4A. In an earlier study it was demonstrated that both wt and mutant Fc hinge fragments are transferred to the fetuses as intact molecules (7). Thus, differences in transfer do not appear to be due to differences in susceptibility to proteolysis of wt vs mutant molecules. The transmission of I253A, H310A, and H435A mutants was only approximately 10 to 20% that of the wt Fc hinge or H285A, demonstrating the central role played by these residues in the maternofetal transfer of IgG. Thus, these mutations have similar effects on maternofetal transfer and catabolism. However, the correlation between the β -phase half-life and maternofetal transmission found for I253A, H310A, H433A, and H435A was not observed for the H436A mutant. Relative to wt Fc hinge, this mutant has a similar activity in maternofetal transmission but a shorter half-life (Table II and Fig. 4A); possible reasons for this are discussed more fully below.

FIGURE 3. Elimination curves of recombinant Fc hinge fragments. The percentage of initial radioactivity (logarithmic scale) is plotted vs time post-initial injection of radiolabeled Fc hinge fragment. Representative curves for one mouse from each group are shown.

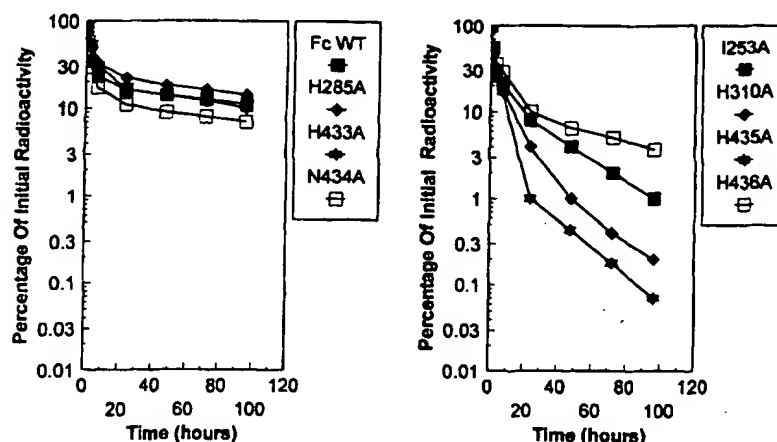


Table II. Catabolism of recombinant Fc-hinge fragments in BALB/c mice

Fc-hinge Fragment	No. of Mice	α -Phase Half-Life	β -Phase Half-life
WT Fc-hinge	5	11.9 ± 0.2	119.1 ± 11.5
I253A	11	9.0 ± 1.8	26.2 ± 1.9
H285A	6	10.8 ± 3.0	106.4 ± 11.7
H310A	9	5.8 ± 0.2	16.8 ± 1.2
H433A	9	9.6 ± 1.0	114.8 ± 10.8
H433A/N434Q	9	9.4 ± 1.1	76.9 ± 10.3
N434A	4	9.0 ± 0.2	110.0 ± 9.2
N434Q	5	11.7 ± 0.3	115.0 ± 12.6
H435A	6	4.4 ± 0.2	17.4 ± 2.8
H436A	9	8.5 ± 0.3	48.7 ± 2.4

Intestinal transfer

The intestinal transmission of recombinant Fc hinge derivatives was analyzed by measuring their ability to inhibit the transfer of radiolabeled mIgG1 across the intestinal barrier of neonatal mice (Fig. 4B). The results are consistent with the data obtained for maternofetal transmission of the I253A, H310A, and H435A mutants, indicating that the same receptor and mechanism of transmission are involved in both transcytotic processes. However, the H436A mutant is transferred across the maternofetal barrier of SCID mice almost as efficiently as the wt Fc hinge (Fig. 4A) and yet does not inhibit the transfer of mIgG1 across the neonatal intestine as effectively. Thus, for this mutant, the half-life and inhibition of neonatal transfer are reduced relative to the wt Fc hinge, and yet maternofetal transfer appears to be unaffected.

Affinity for FcRn

The relative affinities of the recombinant Fc hinge fragments for binding to recombinant mFcRn were estimated by measuring their ability to inhibit binding of [125 I]FcRn to mIgG1-Sepharose (Fig. 5A). The data demonstrate that in all cases, the mutants with short half-life and decreased activity in transcytosis assays (maternofetal and neonatal) also have a lower affinity for binding to FcRn, with the exception of H436A. Despite a lower relative affinity for FcRn, this mutant is transferred across the maternofetal barrier as efficiently as wt Fc hinge, and yet has a reduced serum half-life and activity in intestinal transfer assays.

Binding to SpA

It has been previously shown that the SpA binding site and the catabolic site are located at the CH2-CH3 domain interface of

mIgG1 (4), and therefore, the effect of mutations on the binding of the Fc hinge fragments to SpA were analyzed in direct binding studies (Fig. 5B). The data indicate that H310A and H435A are greatly impaired in SpA binding (9–12% of wt), whereas mutation of I253 or H433 has a less marked effect (30–35% of wt).

Discussion

This study demonstrates that amino acid residues of the CH2 domain (I253 and H310) and CH3 domain (H435 and, to a lesser extent, H436) are involved in regulating the transcytosis and serum persistence of mIgG1. Although this conclusion is drawn from the analysis of recombinant Fc hinge fragments that are expressed in an aglycosylated form, our earlier studies demonstrated that the wt Fc hinge fragment has the same β -phase half-life (4) and activity in transcytosis assays (6) as complete glycosylated mIgG1. This indicates that for this isotype it is valid to extend studies with aglycosylated Fc hinge fragments to complete IgGs. Thus, residues in both domains play a key role in the two processes that involve FcRn-mIgG1 interactions, and the locations of these amino acids on the three-dimensional structure of the homologous human Fc γ 1 (17) are shown in Figure 6. This conclusion may appear to contradict our earlier statement that mutations in the CH2 domain have a more marked effect than mutations in the CH3 domain (4–7). However, in these earlier studies only the effect of simultaneous mutation of both H433 and N434 on transcytosis/catabolism was analyzed, and this indicated a minor role for these residues. In the current analysis, mutation of each of these two amino acids individually has insignificant effects on both transcytosis and catabolism. The most plausible explanation for the observed effects of mutation of both H433 and N434 is that simultaneous mutation of these two amino acids causes a local perturbation in the orientation of the adjacent histidine (H435), which, in contrast to H433 and N434, plays a critical role in the FcRn-mIgG1 interaction.

Further analysis of the region encompassing H310 and Q311, which had previously been analyzed in the context of simultaneous mutation of H310, Q311 to A310, N311, demonstrates the central role of H310 in the FcRn-mIgG1 interaction. Mutation of H310 to alanine has an effect that is as marked as that seen for the H310A/Q311N mutant analyzed earlier, and for this reason the effect of mutation of Q311 alone was not investigated in the current study. In contrast to I253, H310, and H435, H436 plays a more minor role in maintaining serum IgG levels and transcytosis. Both I253 and H310 are highly conserved in all murine and human IgG isotypes (8), whereas H435 and H436 show a lesser degree of conservation (Y435, Y436 in mIgG2b; L436 in a mIgG2a allotype; Y436 in

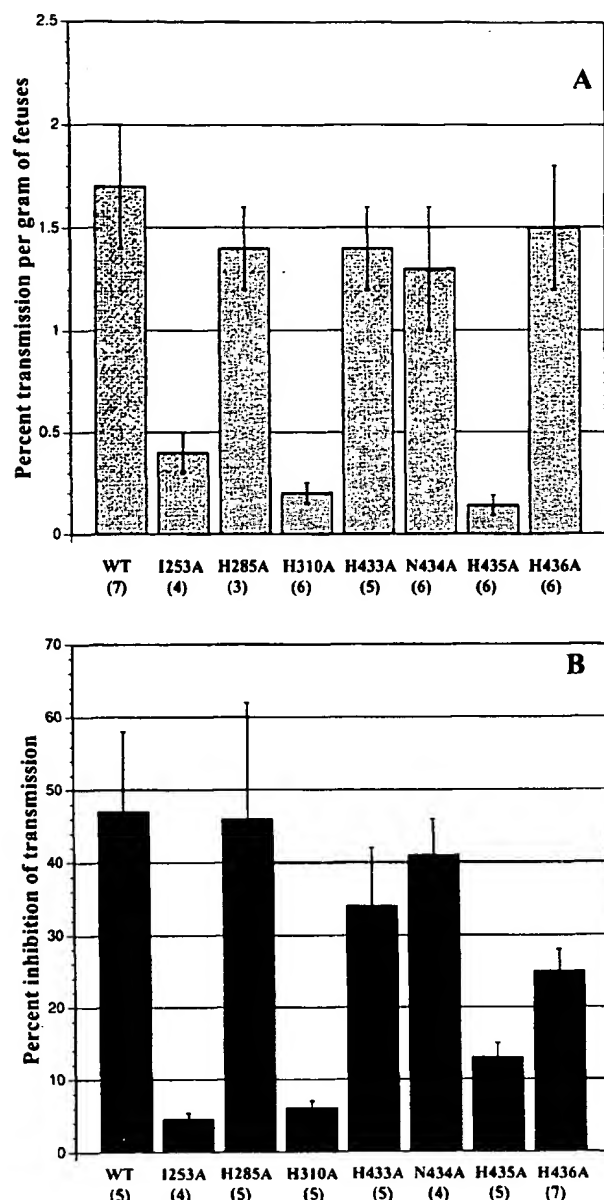


FIGURE 4. Transcytosis of recombinant Fc hinge fragments. The numbers in parentheses represent the number of mice used for each experiment. *A*, Maternofetal transmission of recombinant Fc hinge fragments in SCID mice. *B*, Inhibition of intestinal transmission of radiolabeled mIgG1 by recombinant Fc hinge fragments in BALB/c neonates. The value for H433A is not significantly different from that for wt Fc hinge (by Student's test, $p = 0.127$).

human IgG1, IgG2, and IgG4; F436 in human IgG3 and R435 in a human IgG3 allotype). These sequence differences might account for the shorter serum half-lives of mIgG2b and human IgG3 (19) relative to other IgG isotypes. To date, there are no consistent data available concerning the relative transcytotic activities of different human (20) and mouse IgG (16) isotypes, and therefore, it is not possible to hypothesize about the effects of amino acid differences at positions 435 and 436 on maternofetal or neonatal transfer. In contrast to IgGs, IgM, IgE, and IgA have short serum half-lives and are not transferred across the placental/yolk sac barrier or neonatal gut (21, 22). Consistent with these observations, none of the residues shown in this study to be important for mediating the Fc-FcRn interaction are present in IgM, IgE, and IgA, although

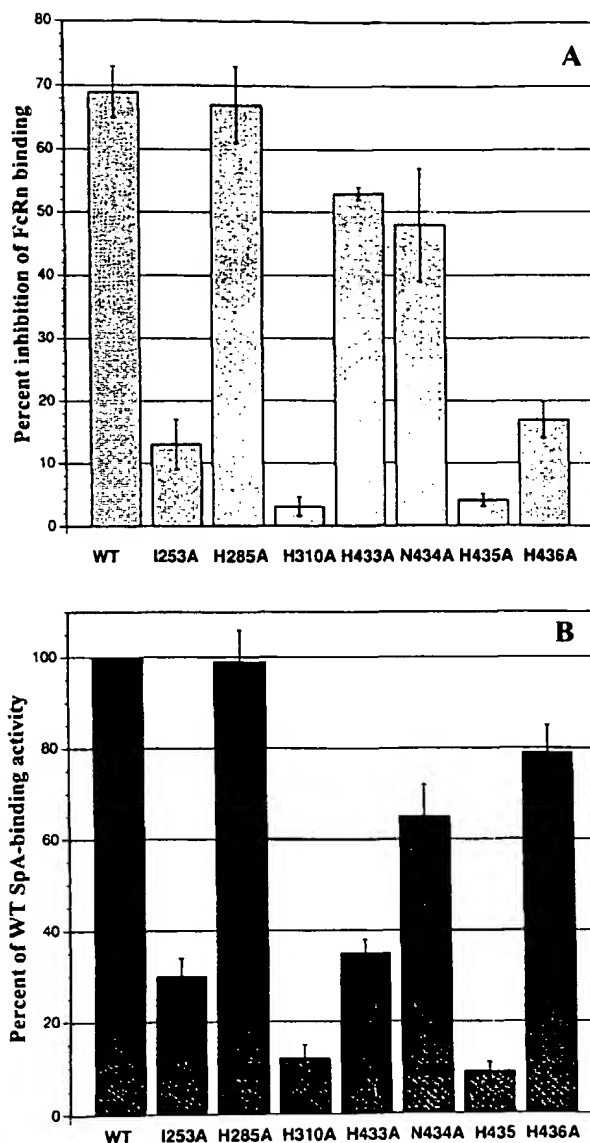


FIGURE 5. Binding of recombinant Fc hinge fragments to FcRn and SpA. *A*, Percent inhibition of FcRn binding to mIgG1-Sepharose relative to binding in absence of inhibitor (average of three separate experiments). *B*, Percentage of Fc hinge fragment binding to SpA-Sepharose relative to the binding of wt Fc hinge (average of three separate experiments).

these three Ig classes of both humans and mice share significant homology with IgGs in other regions of the respective molecules (8).

I253 is a highly exposed, hydrophobic residue that is conserved in all IgG molecules belonging to mammals (8). In the present study we have confirmed that mutation of this isoleucine to alanine results in considerable decreases in the serum half-life and transcytosis across the maternofetal barrier or neonatal intestine. This clearly indicates that I253 fulfills a key physiologic role beyond binding to SpA (17). The amino acid residues flanking I253 are involved in the binding of human Fc to SpA (17), and their participation in the binding of FcRn cannot be excluded. Thus, M252 is highly conserved in all IgG isotypes of mouse, rat, guinea pig, rabbit, and human with a few exceptions, such as mIgG1 and rat IgG1/IgG2a, for which threonine replaces methionine (8). Similarly, position 254 is occupied by serine for all isotypes and species

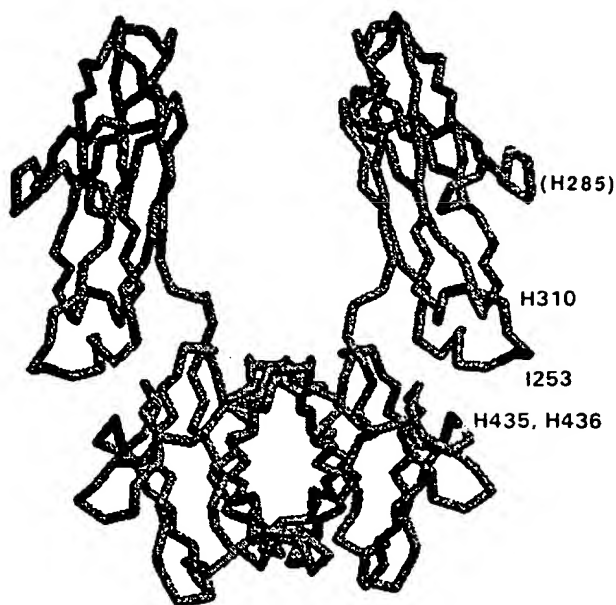


FIGURE 6. The three-dimensional structure of the Fc region of IgG (17), with the amino acids involved in transcytosis and catabolism indicated. The location of H285, which is not involved in interacting with FcRn, is also shown with lettering for H285 in parentheses to indicate that it is not involved in the FcRn-mIgG1 interaction. The program RASMOL (Roger Sayle, Bioinformatics Research Institute, University of Edinburgh, U.K.) was used to display this structure.

except the above-mentioned mouse and rat isotypes that have threonine at this position. These changes in positions 252 and 254 may correlate with longer half-life and more efficient transcytosis of mIgG1 compared with the other isotypes.

For all mutants except H433A and H436A, binding to FcRn and SpA is impaired to a similar degree. H433A has reduced SpA binding relative to wt Fc hinge, but is unaffected when interacting with FcRn. Conversely, the H436A mutation has the opposite effect. Thus, although the SpA and FcRn interaction sites overlap, the overlap is not complete and the "footprints" of SpA and FcRn on mIgG1 are distinct. This is also consistent with the differences in pH dependence that are observed for the FcRn-mIgG1 and SpA-mIgG1 interactions (23–25).

The pH dependence of the interaction between IgG and FcRn (binding at pH 6–6.5 and release at pH 7–7.5) (23, 24) falls in the range of the pK value of the imidazole side chains of histidine. Taken together with the data from this study, this suggests that the marked pH dependence of the IgG-FcRn interaction is determined by the surface accessible histidine residues at positions 310, 435, and 436 located at the interface of the CH2 and CH3 domains. This

is in accord with data of Bjorkman and colleagues indicating that for mIgG2a, there are three titratable residues in the pH range of 6.4 to 6.9 (26). Consistent with these studies (26), analysis of the H310A mutant demonstrates that H310A plays a role in mediating the Fc-FcRn interaction both in vitro and in vivo. In contrast, however, analysis of H433A and H435A shows that for mIgG1, mutation of H435 to alanine results in a loss of affinity for FcRn, whereas H433 does not play a role in FcRn binding. Furthermore, mutation of H436 to alanine results in an Fc hinge fragment that has reduced affinity for FcRn. Thus, the histidines that play a role in mediating the high affinity of the mIgG1-FcRn interaction are H310, H435, and, to a lesser extent, H436. The reasons for the apparent differences in H433 and H435 between our data and those of others (26) are not clear, but in the latter study different isotypes (mIgG2a, mIgG2b, and human IgG4) with consequent sequence differences in the residues both at and in proximity to the FcRn interaction site were used. Thus, it is conceivable that in the context of differences in the sequences of surrounding residues, the relative roles of H433 and H435 are distinct in different isotypes.

The close correlation between the effect of mutations of the Fc hinge fragments on pharmacokinetics, transcytosis across neonatal brush border/yolk sac, and affinity for FcRn (Table III) supports the concept that FcRn is involved in all these processes (2, 3). This is also consistent with experiments showing that in mice lacking FcRn due to loss of β_2m expression, IgGs have decreased intestinal transmission (1, 27) and abnormally short serum half-lives (2, 3). For both the control of catabolism and transcytosis, it has been hypothesized that only the IgG molecules bound to FcRn are protected from degradation and reenter the circulation (catabolism) or traverse the yolk sac/neonatal intestine (transcytosis) (28). FcRn was first identified as a functional protein in tissues of different species (placenta, yolk sac, and brush border of neonatal intestine) involved in the transmission of Ab from mother to fetus or neonate (23, 24, 29–32). More recently, mouse FcRn α -chain mRNA has been isolated from organs not involved in maternal transmission of IgGs, such as liver, lung, heart, and spleen (2). Rat and human homologues of FcRn have also been found to be ubiquitously expressed at the mRNA level (33–35). This strongly suggests that FcRn might be synthesized by the endothelial cells within these organs. Consistent with this, FcRn α -chain mRNA (2) and the corresponding protein (J. Borvak et al., unpublished observations) have been isolated from cultivated mouse endothelial cells (SVEC), suggesting that endothelial cells might be the site of IgG catabolism. The isolation of a human homologue of FcRn from human placenta (30–32) that is ubiquitously expressed in adult tissues (33) together with the high degree of conservation of I253, H310, and H435 in human IgGs (8) indicate that the same mechanisms of maternofetal transfer and homeostasis of serum IgGs are operative in humans. Understanding these processes in molecular

Table III. Pearson's correlation coefficient test

	Catabolism	Intestinal Transfer	Affinity for FcRn	Protein A Binding
Maternofetal Transmission	$r = 0.8703$	$r = 0.8928$	$r = 0.8358^a$	$r = 0.8838$
Catabolism	$p = 0.0049$	$p = 0.0028$	$p = 0.0097$	$p = 0.0036$
		$r = 0.9450$	$r = 0.9776$	$r = 0.7107$
		$p = 0.0004$	$p = 0.00003$	$p = 0.0482$
Intestinal Transfer			$r = 0.9531$	$r = 0.8361$
			$p = 0.00025$	$p = 0.0097$
Affinity for FcRn				$r = 0.7709$
				$p = 0.0251$

Correlation coefficient excluding values obtained for H436A; $r = 0.9917$; $p = 0.00001$.

detail has implications both for the modulation of the pharmacokinetics of therapeutic IgGs and for the enhancement of maternofetal transfer of IgGs that might be of value in passive immunization of fetuses.

The pH dependence of the FcRn-IgG interaction (23, 24) suggests that the subcellular site (cell surface or intracellular compartment) at which binding occurs will differ for neonatal transcytosis and maternofetal transfer/control of catabolism, as discussed previously (2). Other unknown factors, such as the rate of recycling in these different cellular compartments, may also play a role in determining the effective concentration of FcRn. These differences between the processes and the cell types involved, despite the involvement of a common receptor, may explain the behavior of the H436A mutant, for which the half-life, intestinal transfer, and affinity for FcRn do not correlate with the maternofetal transmission as closely as for the other mutants. A further explanation for the anomalous effects of the H436A mutation might be as follows; mutation of H436 to alanine does not have as marked an effect on catabolism, inhibition of intestinal transfer, and binding to FcRn as those observed for I253A, H310A, and H435A, and in contrast to the other three assays, the maternofetal transfer assay is conducted in the absence of competition by endogenous IgGs using SCID mice. Thus, in this situation, the effect of this mutation on maternofetal transfer might only manifest itself if an analysis of the time course of transmission is conducted or if transfer is analyzed in the presence of endogenous competing IgGs in, for example, BALB/c mice. In contrast, for mutants such as I253A, H310A, and H435A that have lower affinity than H436A for binding to FcRn in competition assays, the low activities in all three *in vivo* assays (catabolism, maternofetal transfer, and inhibition of neonatal transcytosis) correlate closely.

In summary, this study has resulted in the unequivocal identification of a role for three highly conserved histidines of mIgG1 (H310, H435, and, to a lesser degree, H436) in the control of catabolism and maternofetal/neonatal transcytosis. Thus, taken together with earlier data implicating I253 in these processes, these residues are critical for the FcRn-mIgG1 interaction. This study extends further the evidence in support of the involvement of FcRn in both transcytosis and catabolism, and has relevance to understanding the molecular mechanisms that regulate these essential functions of IgGs.

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References

- Israel, E. J., V. K. Patel, S. F. Taylor, A. Marshak-Rothstein, and N. E. Simister. 1995. Requirement for a β_2 -microglobulin associated Fc receptor for acquisition of maternal IgG by fetal and neonatal mice. *J. Immunol.* 154:6246.
- Ghetie, V., J. G. Hubbard, J. K. Kim, M. F. Tsen, Y. Lee, and E. S. Ward. 1996. Abnormally short serum half-lives of IgG in β_2 -microglobulin deficient mice. *Eur. J. Immunol.* 26:690.
- Junghans, R. P., and C. L. Anderson. 1996. The protection receptor for IgG catabolism is the β_2 -microglobulin-containing neonatal intestinal transport receptor. *Proc. Natl. Acad. Sci. USA* 93:5512.
- Kim, J. K., M. F. Tsen, V. Ghetie, and E. S. Ward. 1994. Identifying amino acid residues that influence plasma clearance of mouse IgG1 fragments by site directed mutagenesis. *Eur. J. Immunol.* 24:542.
- Kim, J. K., M. F. Tsen, V. Ghetie, and E. S. Ward. 1994. Catabolism of the murine IgG1 molecule: evidence that both CH2-CH3 domain interfaces are required for persistence of IgG1 in the circulation of mice. *Scand. J. Immunol.* 40:457.
- Kim, J. K., M. F. Tsen, V. Ghetie, and E. S. Ward. 1994. Localization of the site of the murine IgG1 molecule that is involved in binding to the murine intestinal Fc receptor. *Eur. J. Immunol.* 24:2429.
- Medesan, C., C. Radu, J. K. Kim, V. Ghetie, and E. S. Ward. 1996. Localization of the site of the IgG molecule that regulates maternofetal transmission in mice. *Eur. J. Immunol.* 26:2533.
- Kabat, E. A., T. T. Wu, H. M. Perry, K. S. Gottesman, and C. Foeller. 1991. *Sequences of Proteins of Immunological Interest*. U.S. Department of Health and Human Services, Washington, DC.
- Popov, S., J. G. Hubbard, J. K. Kim, B. Ober, V. Ghetie, and E. S. Ward. 1996. The stoichiometry and affinity of interaction of murine Fc fragments with the MHC class I-related receptor, FcRn. *Mol. Immunol.* 33:521.
- Horton, R. M., H. D. Hunt, S. N. Ho, J. K. Pullen, and L. R. Pease. 1989. Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. *Gene* 77:61.
- Carter, P., H. Bedouelle, and G. Winter. 1985. Improved oligonucleotide site-directed mutagenesis using M13 vectors. *Nucleic Acids Res.* 13:4431.
- Kunkel, T. A. 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc. Natl. Acad. Sci. USA* 82:488.
- Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain terminating inhibitors. *Proc. Natl. Acad. Sci. USA* 74:5463.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680.
- Fraker, P. J., and J. C. Speck. 1978. Protein and cell membrane iodinations with a sparingly soluble chloroamide, 1,3,4,6-tetrachloro-3a,6a-diphenylglycouril. *Biochem. Biophys. Res. Commun.* 80:849.
- Guyer, R. L., M. E. Koshland, and P. M. Knopf. 1976. Immunoglobulin binding by mouse intestinal epithelial cell receptors. *J. Immunol.* 117:587.
- Deisenhofer, J. 1981. Crystallographic refinement and atomic models of human Fc fragment and its complex from fragment B of protein A from *Staphylococcus aureus* at 2.9 and 2.8 Å resolution. *Biochemistry* 20:2361.
- Mariani, G., and W. Strober. 1991. Immunoglobulin metabolism. In *Fc Receptors and the Action of Antibodies*. H. Metzger, ed. American Society for Microbiology, Washington, DC, p. 94.
- Ward, E. S., and V. Ghetie. 1995. The effector functions of immunoglobulins: implications for therapy. *Ther. Immunol.* 2:77.
- Mellbye, O. J., and J. B. Natvig. 1973. Presence and origin of human IgG subclass proteins in newborns. *Vox Sang.* 24:206.
- Zuckier, L. S., L. D. Rodriguez, and M. D. Scharff. 1989. Immunologic and pharmacologic concepts of monoclonal antibodies. *Semin. Nucl. Med.* 29:166.
- Wild, A. E. 1973. Transport of immunoglobulin and other proteins from mother to young. In *Lysosomes in Biology and Pathology*, Part 3. J. T. Dingle, ed. North Holland, Amsterdam, p. 179.
- Wallace, K. H., and A. R. Rees. 1980. Studies on the immunoglobulin-G Fc fragment receptor from neonatal rat small intestine. *Biochem. J.* 188:9.
- Rodewald, R., and J. P. Kraehenbuhl. 1984. Receptor-mediated transport of IgG. *J. Cell Biol.* 99:159s.
- Ey, P. L., S. J. Prowse, and C. R. Jenkin. 1978. Isolation of pure IgG1, IgG2a and IgG2b immunoglobulins from mouse serum using protein A-Sepharose. *Immunochimistry* 15:429.
- Raghavan, M., V. R. Bonagura, S. L. Morrison, and P. J. Bjorkman. 1995. Analysis of the pH dependence of the neonatal receptor/immunoglobulin G interaction using antibody and receptor variants. *Biochemistry* 34:14649.
- Zijlstra, M., M. Bix, N. E. Simister, J. M. Loring, D. H. Raulet, and R. Jaenisch. 1990. β_2 -Microglobulin deficient mice lack CD4⁺8⁺ cytolytic T cells. *Nature* 344:742.
- Brambell, F. W. R., W. A. Hemmings, and I. G. Morris. 1964. A theoretical model of gammaglobulin catabolism. *Nature* 201:1352.
- Roberts, D. M., M. Guenther, and R. J. Rodewald. 1990. Isolation and characterization of the Fc receptor from the fetal yolk sac of the rat. *J. Cell Biol.* 111:1867.
- Simister, N. E., C. M. Story, H.-L. Chen, and J. S. Hunt. 1996. An IgG-transporting Fc receptor expressed in the syncytiotrophoblast of human placenta. *Eur. J. Immunol.* 26:1527.
- Kristoffersen, E. K., and R. Matre. 1996. Co-localisation of the neonatal Fc receptor and IgG in human placental term syncytiotrophoblast. *Eur. J. Immunol.* 26:1668.
- Leach, J. L., D. D. Sedmark, J. M. Osborne, B. Rahill, M. D. Lairmore, and C. L. Anderson. 1996. Isolation from human placenta of the IgG transporter, FcRn, and localization to the syncytiotrophoblast. *J. Immunol.* 157:3317.
- Story, C. M., J. E. Mikulska, and N. E. Simister. 1994. A major histocompatibility complex class I-like Fc receptor cloned from human placenta: possible role in transfer of immunoglobulin G from mother to fetus. *J. Exp. Med.* 180:2377.
- Simister, N. E., and K. E. Mostov. 1989. Cloning and expression of the neonatal rat intestinal Fc receptor, a major histocompatibility complex class I antigen homolog. *Cold Spring Harb. Symp. Quant. Biol.* 54:517.
- Blumberg, R. S., T. Koss, C. M. Story, D. Barisani, J. Polischuk, A. Lipin, L. Pablo, R. Green, and N. E. Simister. 1995. A major histocompatibility complex class I-related Fc receptor for IgG on rat hepatocytes. *J. Clin. Invest.* 95:2379.